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# **Plasma Volume Methodology: Evans Blue, Hemoglobin - Hematocrit, and Mass Density Transformations**

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## SUMMARY

Detailed descriptions of methods for measuring absolute levels and changes in plasma volume are presented along with derivations of pertinent equations. Reduction in variability of the Evans blue dye dilution technique using chromatographic column purification suggests that the day-to-day variability in the plasma volume in humans is less than  $\pm 20$  ml. Mass density determination using the mechanical-oscillator technique provides a method for measuring vascular fluid shifts continuously for assessing the density of the filtrate, and for quantifying movements of protein across microvascular walls. Equations for the calculation of volume and density of shifted fluid are presented.

## INTRODUCTION

Determination of plasma volume (PV) with Evans blue dye (T-1824) linked to albumin and measured spectrophotometrically was first reported by Gregersen (ref. 1-3). This technique was developed primarily for the Armed Forces. Essentially the same technique, with some refinement on the separation and analysis of the dye, is in use today. The accuracy and reliability of this dye method depends upon the stability of plasma albumin content over the period of measurement, as well as upon the kinetics of the bond between the dye and albumin molecule. Similar results are obtained when the Evans blue technique is compared with the radioiodinated (I-131) labeled albumin technique (refs. 4-7). When the PV is determined from analysis of one 10-min postinjection dye sample, good agreement is obtained with the zero-time extrapolation technique, using multiple postinjection blood samples (ref. 6). Evans blue dye subjected to the Ames test for carcinogenicity (refs. 8 and 9) gave negative results (ref. 10).

With the advent of radioactive tracers, PV has usually been measured with one of the iodine isotopes (I-125, I-131). The problem then arises of the appropriateness of using even tracer quantities of radioisotopes in humans in nonlife-threatening situations. It is possible that "the risks of low levels of radiation are much greater than linear extrapolations would indicate" (ref. 11).

For these reasons we wish to reemphasize the usefulness of Evans blue dye for the measurement of PV and to present a technique slightly modified from that of Campbell, Frohman, and Reeve (ref. 7) for analyzing the dye concentration.

A second problem is the measurement of changes in PV over minutes and hours. Repeated dye injections result in saturation of the albumin-binding sites, creating such high preinfusion plasma concentrations that injection of additional dye cannot

be measured accurately. As a result, the short-term (hourly) changes in plasma volume have been calculated from sequential measurements of hematocrit (Hct) and hemoglobin (Hb) concentrations (refs. 12-14) and the Hct alone (refs. 15-17). In addition to the errors incurred in the measurement of Hct and Hb, and the probable error in using antecubital venous blood values as representative of total blood volume levels, the act of drawing blood samples through a catheter may cause a change in vascular hemodynamics and change the Hct and Hb concentrations. In spite of these probable sources of error, use of the Hb-Hct and Hct equations for calculating changes in PV have proven useful and will continue to be used. Derivations of these equations are presented.

Recently, the development of instruments (ref. 18) for very accurate measurement of blood and plasma density has made possible not only a fast and precise method for measuring changes in these volumes from discrete blood samples (refs. 19 and 20), but it also provides for the first time a continuous measurement of changes in PV (refs. 21-23). This measurement technique is also described fully.

## EVANS BLUE DYE AND ISOTOPE LABELING

### Evans Blue Dye and I-131 Labeled Albumin

Gregersen and associates (ref. 2) were the first to use Evans blue dye (first obtained from Dr. H. M. Evans) to measure PV and, subsequently, they developed the method in detail (refs. 1 and 3). Measurement of dye diluted in untreated plasma results in rather large errors caused by lipemia or other interfering substances which absorb at 615 nm. Allen and colleagues (refs. 24 and 25) were probably the first to use a detergent, which displaces the dye from albumin, and a cellulose pulp matrix to absorb the unbound dye. This basic technique was refined by Campbell et al. (ref. 26) and forms the basis for our technique, which is described here in detail.

#### Reagents:

1. Disodium hydrogen phosphate,  $\text{Na}_2\text{HPO}_4$  (anhydrous): 2% solution (20 g/1000 ml).
2. Teepol-phosphate [Teepol 610 concentrate, Particle Data Laboratories, Ltd., 115 Hahn St., Elmhurst, IL 60126, (312) 832-5658]: Add 20 g  $\text{Na}_2\text{HPO}_4$  to 30 ml Teepol and make up to 1000 ml with distilled water.
3. Potassium phosphate, monobasic,  $\text{KH}_2\text{PO}_4$  (anhydrous): 8% solution (8 g/100 ml).
4. 1:1 Acetone-water solution.
5. Solka Floc BW-40 (60  $\mu$ ) suspension of purified wood cellulose [James River Corp., 650 Main St., Berlin, NH 03570, (603) 752-4600]: Suspend approximately 10 g Solka Floc in 1000 ml 2%  $\text{Na}_2\text{HPO}_4$  (Reagent 1).

6. Evans blue dye (T-1824) standard [Harvey Laboratory and Pharmaceuticals, 113 West Wyoming St., Philadelphia, PA 19140, (215) 457-7600]: Same lot number that is used for injection. Refrigerate. (If in doubt about the lot number being the same, a quantity of dye will always be left over from the vial used for injection.)

#### Preparation of columns:

1. Prepare five columns for each subject: one for the standard, one for the blank using the "pre" sample, and three for the "post" samples. The columns can be fabricated by a glassblower; they are 25 cm long and have a burette stopcock on the lower end of a 13-cm glass tube with a 1-cm inside diameter; attached to the top of this tube is a larger glass tube 7 cm long with a 2.5-cm inside diameter.

2. Insert approximately 100 mg (3 cm) of uncompressed glass wool into each column, wash the tube with distilled water, and pack the glass wool firmly above the small constriction above the stopcock with a large stirring rod to a height of about 0.5 cm.

3. With a pipette (upside down), pipette about 12 ml of the Solka Floc suspension into the column to a height of about 5 cm. (The pipetting should be carried out quickly after shaking the Solka Floc suspension, as it tends to settle very quickly.)

4. Wash each column using a pipette with approximately 10 ml of 2%  $\text{Na}_2\text{HPO}_4$  to pack the column. (If possible, prepare the columns a day in advance of the test so that they will be packed well. When washed well with 2%  $\text{Na}_2\text{HPO}_4$ , the columns can be reused.)

#### Preparation of Evans blue for injection:

1. After the dye has drained to the bottom of the vial, wipe the outside with alcohol and break open. Fill a sterile, 10-ml syringe with the dye, using a sterile 18-gauge needle. Remove the needle and expel all air from the barrel of the syringe. Push the level of the dye to the very tip of the syringe and attach a sterile 20-gauge needle, bevel up. Replace cover over the needle, and label the syringe with the subject's name. Wipe away fingerprints, and weigh the syringe, needle, and cover on an analytical balance. Record this weight to four decimal places.

2. Deliver the loaded and weighed syringe to the person doing the injection. Be sure the needle cover is returned with the empty syringe.

3. Wipe fingerprints from empty syringe and needle, and cover and reweigh the syringe apparatus. Subtract the empty weight (minus the weight of dye in needle) from the filled weight to determine the exact weight of dye injected.

#### Preparation of the plasma samples:

1. Centrifuge the heparinized blood from the "pre" injection and the "post" injection samples (3500 rpm for 10 min) and draw off and save the plasma in an appropriately labeled screw-cap vial.
2. To 1 ml of the "pre" sample in a 50-ml Erlenmeyer flask, add 0.2 ml of the Evans blue standard solution (Reagent 6). Mix by swirling and let stand at least 2 min.
3. Set up the following in 50-ml Erlenmeyer flasks (one for each column):
  - a. "Pre" injection plasma--1 ml (BLANK)
  - b. "Pre" injection plasma--1 ml + standard (STANDARD)
  - c. "Post" injection plasma--1 ml (TEST)
  - d. "Post" injection plasma--1 ml (TEST)
  - e. "Post" injection plasma--1 ml (TEST)
4. Add 15 ml of Teepol-phosphate to each flask, washing any plasma from the sides of the flask; swirl gently for about 15 sec and mix well.

#### Extraction:

1. Transfer the contents of each flask gently onto the Solka Floc in the column with a Pasteur pipette so that its surface is not disturbed.
2. Rinse the flask with 5 ml of Teepol-phosphate and add this volume to the column.
3. When the level of the solution has reached the Solka Floc, add at least 10 ml of 2%  $\text{Na}_2\text{HPO}_4$  to the column. This "wash" must be sufficient to remove all interfering substances, or else cloudiness will result in the final eluate.
4. Allow the "wash" to pass through the column until the fluid is 0.5 cm above the Solka Floc.

#### Elution:

1. Elute immediately.
2. Pipette 0.5 ml of 8%  $\text{KH}_2\text{PO}_4$  into a 10-ml volumetric flask. This phosphate, with the  $\text{Na}_2\text{HPO}_4$  from the column, buffers the pH of the eluate to 7.0.
3. Gently transfer approximately 5 ml of the acetone-water solution into the column and allow it to pass down until the blue front passes through the Solka Floc and nears the bottom.

4. Place the 10-ml volumetric flask with  $K_2PO_4$  under the column and allow the blue front to pass it. (Do not let the top of the column become dry; add more acetone-water if needed.)

5. Allow the columns to stand 15 min before completing the elution.

6. Add 4-5 ml of the acetone-water solution into the column and continue the elution until the eluate fills the flask to the 10-ml mark; all the blue dye should have passed out of the plasma.

7. Read the solutions in a spectrophotometer at 615 nm.

Calculation:

$$\text{Plasma volume (ml)} = \frac{V \times D \times St \times v}{T \times 1.03} \quad (1)$$

where

V = volume (wt) of T-1824 injected

D = dilution of STANDARD (0.2 ml of 1:50; therefore dilution = 1:250)

St = absorbance of STANDARD

v = volume of sample extracted (1 ml)

T = absorbance of TEST sample minus absorbance of BLANK

1.03 = factor introduced to correct for slow dye uptake by the tissues

$$\text{Blood volume} = PV \times \frac{100}{100 - (0.87 \text{ Hct})} \quad (2)$$

where 0.87 is a correction factor (about 4% of the Hct is trapped plasma; therefore, the true volume of packed cells is 0.96 Hct; whole-body Hct is  $0.91 \times$  true packed cell volume; hence,  $0.96 \times 0.91 = 0.87$ ), and Hct is the raw Hct.

Ames Test for Carcinogenicity of T-1824

The Ames assay procedure (refs. 8,9) using Salmonella typhimurium (LT2) strains TA1535, TA1537, TA1538, TA98, and TA100 is about 80% to 90% reliable in detecting carcinogens as mutagens. The test has about the same reliability in identifying chemicals that are not carcinogenic. Therefore, neither a positive nor a negative response proves conclusively that a chemical is or is not carcinogenic to humans. The test consists of comparing the number of histidine-independent colonies on treated agar plates with the number found on control plates. Since all the plated Salmonella indicator organisms undergo a few cell divisions in the presence of the test chemical, the assay procedure must be considered semiquantitative. The establishment of mutagenicity (carcinogenicity) relies on the demonstration of a mutagenic dose-response relationship. The test chemicals are assayed at several dose levels within a nontoxic dose range. Toxicity is present if there is clearing of the background growth "lawn," a decrease in the number of "revertent" colonies below the spontaneous background, or formation of pinpoint colonies of surviving cells.

The test chemicals are considered mutagenic in the Salmonella/microsome preincubation assay if they elicit a reproducible dose-related increase in the number of histidine revertants per plate in one or more of the five test strains. The dye samples (General Diagnostics Lot 4018037 and Harvey Laboratories Lot 10870) were tested initially in strain TA98 up to 200  $\mu$ l/plate, then in all five strains up to 200  $\mu$ l/plate, and, finally, in all five strains up to a dose of 1000  $\mu$ l/plate (approximately 4600  $\mu$ g of anhydrous dye). Each dose for human injection contains 22.6 mg of dye that is diluted into 2.5 to 4.0 liters of plasma. Neither of the two Evans blue dye samples caused any toxic or mutagenic effects in any of the three assay tests.

#### Errors in T-1824 Technique

The repeatability of PV measurements with T-1824 depends upon the constancy of the plasma albumin content over the tracer equilibrium period, and from one measurement period to the next. Results from our studies (refs. 6 and 13) have indicated that: (a) Both T-1824 space and I-131 space, determined from a single sample obtained 10 min after injection, were comparable with the zero-time extrapolation values determined from serial sampling. (b) Four consecutive injections of T-1824 on alternate days, resulting in residual dye buildup to 87% of one dose, did not significantly affect the accuracy of measurement of Evans blue space. (c) Four consecutive injections of I-131 on alternate days resulting in residual radiation buildup of 85% of one dose, did not significantly affect the accuracy of measurement of I-131 space, and (d) T-1824 and I-131 did not interfere with each other when the two tracers were used simultaneously, and their results were comparable (refs. 6 and 13). We have studied the variability of the basal PV (Evans blue space) in normal, healthy humans at various intervals up to 28 days (Table 1). The variability ( $\pm$ SE) between subjects ranges from 76 to 172 ml, whereas the constancy of the PV over 28 days ranges between -31 and +28 ml. The mean ( $\pm$ SE) of the absolute differences between the seven paired measurements is  $8 \pm 7$  ml. These findings emphasize the constancy of the day-to-day PV in men and women living their normal lives (Table 1).

There are two additional problems with the albumin-labeled tracer methods. One involves the constancy of the albumin content over the period of measurement. The net exchange of albumin can vary from 4 to 7% within a 60-min period with subjects at rest or undergoing mild exercise (refs. 27), and there can be an additional shift of albumin (-4% to -8%) during 10 min of maximal exercise (refs. 15,27,28). A comparison was made between the percent change in PV, determined from control and postmaximal-exercise Evans blue spaces, and the change in PV from the Hb-Hct equation. The postexercise Evans blue value was the concentration of residual dye remaining after the single preexercise injection. There was essentially no positive relationship between the PV determined from the two methods, which suggests that analysis of residual dye is useless as an estimate of PV immediately after heavy exercise (ref. 29) and after 8 hr of water immersion (ref. 30). The second problem is that the use of these tracer techniques is limited when one must make repeated measurements of PV over short time intervals. Since the minimum equilibration time



for these tracers is 10 min, PV determinations at shorter intervals are difficult to perform accurately. It is doubtful whether determinations made at consecutive 10-min intervals would be accurate because of the progressively increasing tracer concentrations in the succeeding preinjection baseline samples. Clearly, another analytical technique for estimating changes in PV at shorter intervals is needed.

### Hemoglobin-Hematocrit and Hematocrit Transformations

It is interesting that the early methods for estimating PV and total blood volumes involved the measurement of Hb concentration before and after the injection of hemoglobin solutions (ref. 31). The relative constancy of the number of red blood cells and their content of Hb provides the basis for two related alternative methods for calculating changes in PV over short-term intervals (hours). They involve the measurement of the Hb concentration and Hct (the ratio of the packed cell volume to the cell volume plus PV), and measurement of the Hct alone.

The first equation for the calculation of changes in PV using the Hb and Hct was published by Elkinton, Danowski, and Winkler (ref. 12):

$$\frac{PV_2}{PV_1} = \frac{(1 - Hct_2)}{(1 - Hct_1)} \times \frac{Hb_1}{Hb_2} \quad (3)$$

rewritten as

$$PV_a = PV_b \frac{Hb_b}{Hb_a} \times \frac{(1 - Hct_a)}{(1 - Hct_b)} \quad (4)$$

Strauss et al. (49) put it in the form of percent change:

$$\frac{PV_2}{PV_1} \times 100 = \frac{Hgb_1}{Hgb_2} \times \frac{(1 - Hct_2)}{(1 - Hct_1)} \times 100 \quad (5)$$

rewritten as

$$\% \Delta PV = 100 \frac{Hb_b}{Hb_a} \times \frac{(1 - Hct_a)}{(1 - Hct_b)} \quad (6)$$

where a = after fluid shift and b = before fluid shift.

If the right-hand side of equation (6) is subtracted from 100, the percent change in PV is obtained directly. The derivation of the equation is presented in Table 2. In this equation the change in PV is calculated from the Hct, assuming the red cell volume and the  $F_{cell}$  ratio (total body Hct/large vessel Hct) remain

constant and the total blood volume is equal to the sum of the cell volume and the PV. Use of the Hb concentration corrects for changes in red cell water content, i.e., the mean corpuscular volume.

If the red cell volume and the  $F_{\text{cell}}$  ratio remain constant, changes in the Hct are a measure of the changes in PV. An equation for calculating percent change in PV from Hct values alone is given by Van Beaumont et al. (ref. 16). It involves a proportionality factor  $[100/(100 - \text{Hct}_b)]$  and the percent difference between the before (b) and after (a) ratios:

$$\% \text{ PV} = \frac{100}{100 - \text{Hct}_b} \times \frac{100(\text{Hct}_b - \text{Hct}_a)}{\text{Hct}_a} \quad (8)$$

There have been numerous reports in which percent changes in Hct, calculated with only the percent difference factor, were equated with percent changes in PV (refs. 32-38). The results indicated that the percent changes in Hct were always smaller than the percent changes in PV. Some explanations for this discrepancy between the two calculated values were a differential rate of change between them (ref. 3), or some unexplained problem with the site of blood sampling (ref. 35). Another explanation is that percent changes in plasma are calculated on the basis of the PV alone, while percent changes in Hct are calculated on the basis of total blood volume, not just the plasma portion (ref. 17). Use of the proportionality factor in equation (7) (Table 2) converts (i.e., reduces) the base from total blood volume to the PV, so now all comparisons are made on the basis of the PV. Thus, any variable measured on the basis of total blood volume (e.g., Hb) can be converted to a PV basis with the Hct proportionality factor, provided the  $F_{\text{cell}}$  ratio is known.

If PV is measured and red cell volume is assumed to be constant, a posttreatment PV can be calculated (ref. 17),  $\text{PV}_a = \text{BV}_a - \text{CV}_b$ , where BV is the blood volume, and CV is the red cell volume, and

$$\text{PV}_a = \frac{[\text{PV}_b / 1 - (\text{Hct}_b \times 10^{-2})] - \text{PV}_b}{\text{Hct}_a \times 10^{-2}} - \frac{\text{PV}_b}{1 - (\text{Hct}_b \times 10^{-2})} - \text{PV}_b \quad (9)$$

Also, if the percent change in PV is calculated in the conventional manner, i.e.,  $\% \Delta \text{PV} = [(\text{PV}_b - \text{PV}_a) / \text{PV}_b] \times 100$ , the percent change in any plasma constituent concentration (e.g., protein) must be calculated with the "after" value in the denominator (ref. 17):

$$\% \Delta \text{Protein} = [(\text{Pro}_b - \text{Pro}_a) / \text{Pro}_a] \times 100 \quad (10)$$

The correlation coefficient between measured PV and PV calculated from the Hct changes with data from five studies from the literature is better than 0.95 (ref. 17). Finally, the percent change in the total content (Co) of a plasma constituent can also be calculated from the before-and-after Hct values and the

concentrations (Cn) of the constituent being measured (ref. 28). The composite equation is as follows (ref. 29):

$$\% \Delta Co = \frac{(Cn_a) - [Hct_a(100 - Hct_b) \times Cn_b]/Hct_b(100 - Hct_a)}{[Hct_a(100 - Hct_b) \times Cn_b]/Hct_b(100 - Hct_a)} \times 100 \quad (11)$$

The major advantage of the Hct transformation is that it requires determination of only the Hct. The deficiencies are that it assumes a constant red blood cell volume or mean corpuscular volume (MCV) and that the venous Hct is representative of the total body Hct. A correction factor of 0.91 is usually applied in resting subjects to correct venous Hct to total body Hct (refs. 3 and 13), but changes in this factor would introduce errors in the calculated volumes (ref. 39). One way to provide some correction for potential changes in the mean corpuscular volume is to combine measurements of Hct with those of red cell hemoglobin. The Hb/Hct is a measure of the mean corpuscular hemoglobin concentration (MCHbCn) (ref. 40) and can be used to estimate changes in the mean corpuscular volume (Hct/red blood cell (RBC) concentration) when the RBC concentration is not available (ref. 41). A stable MCHbCn would also indicate an unchanged MCV because a change in cell volume, resulting from a shift of fluid into the RBC, would change the RBC mass; the Hb concentration would remain unchanged, while the Hct and MCHbCn would change.

#### Comparison of Hct and Hb-Hct Methods for Calculating Percent Change in PV

There is not total agreement among studies for the calculated changes in PV with the Hct versus the Hb-Hct methods under some exercise and environmental conditions. On the basis of results obtained from studies on men undergoing exercise dehydration, thermal dehydration, and submaximal and maximal exercise in a thermo-neutral environment, Costill et al. (refs. 42 and 43) concluded that the changes in PV calculated with the Hct method will be in error unless corrections are made for changes in the mean corpuscular volume. Their data suggest that the MCV is reduced under these conditions because of increases in plasma osmolality. On the other hand, Van Beaumont and Rochelle (ref. 44) found no change in MCV with increases in plasma osmolality of 12%, induced by maximal arm exercise. It seems clear that short-term intensive stress has essentially no effect on MCV as measured by the regression of percent change in PV calculated with the Hct or the Hb-Hct. It is possible that increased osmotic concentrations decrease the MCV *in vivo*, but this hypothesis has not been confirmed (ref. 45). Perhaps there is an upper homeostatic limit, *in vivo*, for increased osmolality beyond which the MCV is unchanged. But a linear relationship is unlikely, because the integrity of the RBC volume would presumably be protected within some well-defined "normal" range of plasma osmotic variability. The positive side of this normal range appears to be 0 to 13 mosmol/kg (ref. 13), and the Hct equation could be used to estimate changes in PV. Within this osmotic range fall values from studies involving maximal exercise short-term (maximal) +G<sub>z</sub> acceleration, 70° tilting, sitting in a hot environment, and submaximal exercise below 50%  $\dot{V}O_{2\max}$  for less than 60 min. The probable effect of longer-duration stress (greater than 1 hr) on changes in MCV suggest factors other

than just an increase in plasma osmolality are involved, because osmotic equilibrium between plasma and RBC probably occurs within minutes, not hours.

While the Hct and Hb-Hct transformations provide a method for estimating changes in PV at much shorter intervals than with Evans blue dye or iodine isotope dilution, they cannot be used to measure changes in PV continuously. Measurement of changes in blood density on-line can be used to estimate changes in plasma or blood volumes continuously. This method will be discussed in the following section.

## BLOOD DENSITOMETRY

### Densitometry on Biologic Fluids

Blood density (BD) measurements were first performed in 1887 with the falling-drop method (ref. 46). In the late 1940s, similar techniques were used to estimate Hct, Hb, and plasma protein concentrations (refs. 47 and 48). But because all early density techniques (refractometry, falling-drop method) were either imprecise or laborious, densitometry had been abandoned for use in physiological research. For physical and industrial research purposes, the reliable mass and volume measurements, often hard to perform with the required accuracy, remained the method of choice for determinations of a sample's mass density, i.e., mass per unit volume.

In 1966 a new method was introduced--the mechanical oscillator technique (MOT)--which was first used for high-precision measurements on homogenous fluids and gases in conjunction with X-ray refraction analysis of molecular structure (ref. 18). Shortly thereafter, the MOT was used to measure the density of beverages, gasoline, tritiated water, inorganic and organic solutions, etc. Recently, its use for continuous measurement on blood (refs. 21-23,49-51) and lymph (ref. 52) was introduced.

The MOT combines the advantages of high accuracy (up to  $10^{-6}$  g/ml); small volumes ( $\geq 50$   $\mu$ l); applicability for continuous recordings; short measuring time ( $\geq 1$  s per measuring cycle in the continuous mode, depending upon the dimensions of the oscillator used); and independence from an external acceleration field, which allows its use in weightless conditions. With the introduction of this method in biology, physiology, and clinical medicine, high-precision density measurements of blood and its constituents have regained their usefulness for biomedical research.

### How the Mechanical Oscillator Technique Works

Mass density determinations with the density-measuring apparatus (DMA) employ the MOT, which is based on the mass-spring principle: the actual resonant frequency ( $f$ ) of an oscillating U-shaped glass tube, comparable to a tuning fork in shape and physical features, is determined according to

$$f = 1/2 \pi \sqrt{c/(M_0 + D \cdot V)} \quad (12)$$

where  $c$  is the constant of elasticity for the glass tube,  $M_0$  is the inertial mass of the empty tube (the oscillator),  $D$  is the density (mass per unit volume), and  $V$  the volume of the sample contained in the tube.

The tube is brought to bending-type oscillations perpendicular to the plane of the U by a variable electromagnetic sinusoidal field, and the movements of its tip are sensed by an optical system. The resonant frequency is tracked by a feedback loop. The oscillation duration is determined precisely for each measuring period by sensing and averaging several hundred single cycles on-line. The length of the measuring period can be preset (e.g., 1, 2, 4, or 8 sec in the case of low-volume DMAs). The MOT is based on high-resolution oscillation time determinations.

Before starting a set of density recordings, the system is calibrated with two media of known density, usually air and water. Then, density values are computed by the built-in microprocessor from the average oscillation time. The density signal can be directly fed to a digital/analog output for continuous recordings. Thus, high-precision density measurements can be made on small volumes of biologic fluids without mass or volume measurements.

The vibration of the DMA's glass tube creates centrifugal forces, the resultant of which is directed toward the tip of the oscillator. Since this force is  $\ll 1$  gram in the normal operating range (ref. 53), no interference in terms of blood sedimentation occurs during BD measurements, particularly in the continuous mode.

Deposition of plasma proteins and corpuscular elements (e.g., thrombocyte aggregates) can be prevented by coating the glass surface with silicon prior to measurement using Surfasil<sup>TM</sup> (Pierce Co.). No difference between continuously determined BD and values from synchronously obtained blood samples could be observed when coated oscillators were used, especially for operation exceeding several hours (ref. 21).

#### Significance of the Mass Density of Biologic Fluids

The density of isotonic fluids at a given temperature is a linear function of the fluid's total protein concentration (refs. 54 and 55); for blood this is approximately

$$BPC = PPC(1 - Hct_R - Hct_W) + MCHbCn \cdot Hct_R + MLPC \cdot Hct_W \quad (13)$$

where BPC is the blood protein concentration, PPC is the plasma protein concentration, MCHbCn is the mean corpuscular hemoglobin concentration, MLPC is the mean leukocyte protein concentration,  $Hct_R$  is the red cell hematocrit, and  $Hct_W$  is the white cell hematocrit (given as volume fraction). The contribution of blood platelets is incorporated into the plasma protein concentration after mild centrifugation (platelet-rich plasma), and into the mean leukocyte protein concentration after intense centrifugation of blood. If hemolysis occurs, the influence of additional Hb on the plasma density can be compensated for by colorimetric means (ref. 56) to determine the "true" plasma density.

The plasma protein and mean corpuscular hemoglobin concentrations are regulated within narrow limits under normal physiological conditions whereas Hct may vary more widely; Hct<sub>w</sub> is usually low (<0.01). Since the mean corpuscular Hb concentration is four to five times greater (300-400 gram/liter) than the plasma protein concentration (60-80 g/l), blood protein and Hct are closely correlated. Consequently, the BD which is a function of the blood protein concentration, is correlated linearly with Hct (refs. 19,20,54). However, PD--at a given temperature and protein concentration--is influenced by plasma lipid, glucose, and urea concentration (ref. 55). Further, the leukocyte density becomes more important in cases of leukocytosis. These confounding factors may diminish the accuracy of densitometry for the determination of protein concentration and Hct, although it remains effective for rough estimations, particularly on a screening basis.

In a single experiment, in which the amount of these potentially confounding factors can be assumed to remain constant, density alterations are a precise measure of plasma protein and Hb concentration shifts. Moreover, the density of fluid shifted between the extravascular and intravascular compartments can be computed, since this "filtrate" density differs from both blood and plasma densities (refs. 21 and 39). Experiments which combine this technique with the advantage of continuous BD measurements reveal new insight not only into the amount but also into the time-course of rapid microvascular fluid exchange (refs. 21,23,50,51). During continuous densitometry, proper compensation of possible thermal deviations becomes most important. Recent studies (refs. 19 and 57) have provided data for corrections of temperature-induced errors in on-line density recordings.

#### Density Shifts With Changes in Posture

Blood volume regulation in humans is seriously challenged by assuming an upright posture. Quiet standing leads to rapid and persistent hemoconcentration, which may result in PV losses in excess of 20%. Calculation of the density of fluid leaving or entering the circulatory system, under isotonic conditions, allows for the assessment of the amount of protein which moves across the capillary walls during orthostatically induced capillary filtration.

The mass density of red cells does not change with alterations of posture in euhydrated man (ref. 58). This observation helps to validate the calculation of the density and volume of fluid shifted between intra- and extravascular compartments, using formulae which assume no net fluid transfer between the corpuscular and plasma compartments. For both inward filtration (from assumption of the supine position after the upright) and outward movement of fluid (upright position after supine), essentially the same fluid density (1008 gram/liter, 37°C) is found with an unchanged  $F_{\text{cell}}$  ratio. This result indicates a protein concentration of  $\approx 30$  g/l in the shifted fluid, or approximately 40% of that in the blood plasma (39).

## Continuous Blood Density Measurements

The MOT has been employed for continuous measurements on arterial blood in animals (refs. 21,23,50) and during blood dialysis in patients (refs. 23 and 49). Recently, coronary sinus blood in dogs was used for on-line density recordings (ref. 51). The accuracy of the density data was better than  $1 \cdot 10^{-4}$  g/ml in these studies.

Changes in the plasma protein concentration after blood volume alterations provide an accurate measure of volume replacement only if the net protein concentration of the shifted fluid is known. Results from different hemorrhage studies are contradictory and demonstrate that filtrate protein content ranges from zero to about half of the plasma concentration after hemorrhage. If a significant amount of protein moves with the filtrate, then the degree of fluid replacement is underestimated from calculations which are based on the assumption that the observed plasma protein concentration changes are due to an admixture of protein-free fluid. Continuous monitoring of the mass density of flowing blood, combined with plasma density and Hct determinations, can help to elucidate the volume and protein concentration of even small fluid shifts. Results from a recent study on the effects of moderate hemorrhage (ref. 21) suggested that either the inward-shifted fluid had a higher density than normal ultrafiltrate, and/or there was a rise of the ratio of whole-body to large-vessel Hct ( $F_{\text{cell}}$  ratio).

The investigation of rapid compensatory mechanisms after blood loss is complicated by the possibility of simultaneous shifts in the  $F_{\text{cell}}$  ratio. If it is altered, the observed changes in measured Hct are due only in part to transcapillary fluid flux, and volume shift results from the Hct data may be in error.

If the  $F_{\text{cell}}$  ratio is constant, as during passive body tilting, the density of transcapillary exchanged fluid can be calculated from blood density (BD) and plasma density (PD) changes (refs. 21 and 39) using the formulae:

$$FD = Pd_0 - \frac{Hct'(1 - Hct_0)}{Hct_0 - Hct'} (PD_0 - PD') \quad (14)$$

or

$$FD = BD_0 - \frac{Hct'}{Hct_0 - Hct'} (BD_0 - BD') \quad (15)$$

where  $PD_0$ ,  $Hct_0$ , and  $BD_0$  refer to the state with the larger blood volume (after volume repletion, or before outward shift), whereas  $PD'$ ,  $Hct'$ , and  $BD'$  refer to the hemoconcentrated condition. Hct is corrected for whole body hematocrit. Provided there are no measuring errors and the red cell volume remained constant, the fluid density (FD) from both equations should be identical.

Whereas plasma density and Hct cannot be measured continuously, the mechanical oscillator technique provides the advantage of precise on-line measurement of blood density changes. As an example, figure 1 shows the first blood density curve

measured in a human which was obtained from venous (cubital) blood when the subject changed from the sitting to the standing and the supine positions. There are transient deviations from a simple exponential time-course curve immediately after the postural changes which may be caused by baroreflex-driven dynamic resettlings of the peripheral resistance, thus influencing the capillary filtration pressure on a minute-by-minute basis, in addition to the normal hydrostatic effects on microvascular fluid exchange.

From the magnitude of the density alterations, the volume of the exchanged fluid (FV) can be calculated from

$$FV = \frac{BD_0 - BD'}{BD' - FD} \quad (\text{fraction of } BV_0) \quad (16)$$

Thus, with use of blood densitometry, not only the magnitude, but also the continuous time-course of fluid shifts can be detected. Use of this technique should result in significant advances in our knowledge of fluid-electrolyte-protein physiology.



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TABLE 1. Variability ( $\bar{X} \pm \text{SE}$ ) of Evans blue space (plasma volume) in normal males (m, 18-32 yr) and females (f, 30-41 yr) in the basal state in 3- to 27-day periods.  $[\Delta] = \bar{X}_{\text{Day}_n} - \bar{X}_{\text{Day}_1}$

Subj.	Day 1	Day 4	Day 8	Day 14	Day 24	Day 28
12 (m)	3531 $\pm 80$	3556 [+25] $\pm 79$				
17 (m)	3489 $\pm 76$	3461 [-28] $\pm 81$				
19 (m)	3112 $\pm 120$		3115 [+3] $\pm 105$			
7 (m)	3453 $\pm 160$			3459 [+6] $\pm 157$		3422 [-31] $\pm 172$
23 (m)	2971 $\pm 97$			2989 [+18] $\pm 90$		
7 (f)	2678 $\pm 98$				2683 [+5] $\pm 97$	

TABLE 2. Derivation of the hemoglobin-hematocrit equation for calculation of change in plasma volume

Basic equations:	where:	PV = plasma volume
		CV = corpuscular volume
(i) $PV = BV - CV$		BV = (total) blood volume
(ii) $CV = BV \times Hct$		Hct = hematocrit, whole body (red + white)
		Hb = blood hemoglobin concentration
(iii) $BV_a = BV_b \times \frac{Hb_b}{Hb_a}$		a = after fluid shift
(assuming unchanged		b = before fluid shift
$F_{cell}$ ratio)		
$\Delta PV = \frac{PV_a - PV_b}{PV_b}$	(fraction of $PV_b$ )	
(eq. i) $\Delta PV = \frac{(BV_a - CV_a) - (BV_b - CV_b)}{BV_b - CV_b}$		
(eq. ii) $\Delta PV = \frac{BV_a - (BV_a \times Hct_a) - [BV_b - (BV_b \times Hct_b)]}{BV_b - (BV_b \times Hct_b)}$		
$\Delta PV = \frac{BV_a(1 - Hct_a) - BV_b(1 - Hct_b)}{BV_b(1 - Hct_b)}$		
(eq. iii) $\Delta PV = \frac{BV_b \times (Hb_b/Hb_a)(1 - Hct_a) - BV_b(1 - Hct_b)}{BV_b(1 - Hct_b)}$		
$\Delta PV = \frac{(Hb_b/Hb_a)(1 - Hct_a) - (1 - Hct_b)}{1 - Hct_b} = \frac{Hb_b}{Hb_a} \times \frac{1 - Hct_a}{1 - Hct_b} - 1$		
$\% \Delta PV = 100 \left[ \frac{Hb_b}{Hb_a} \times \frac{1 - Hct_a}{1 - Hct_b} \right] - 100$	(Eq. 7)	

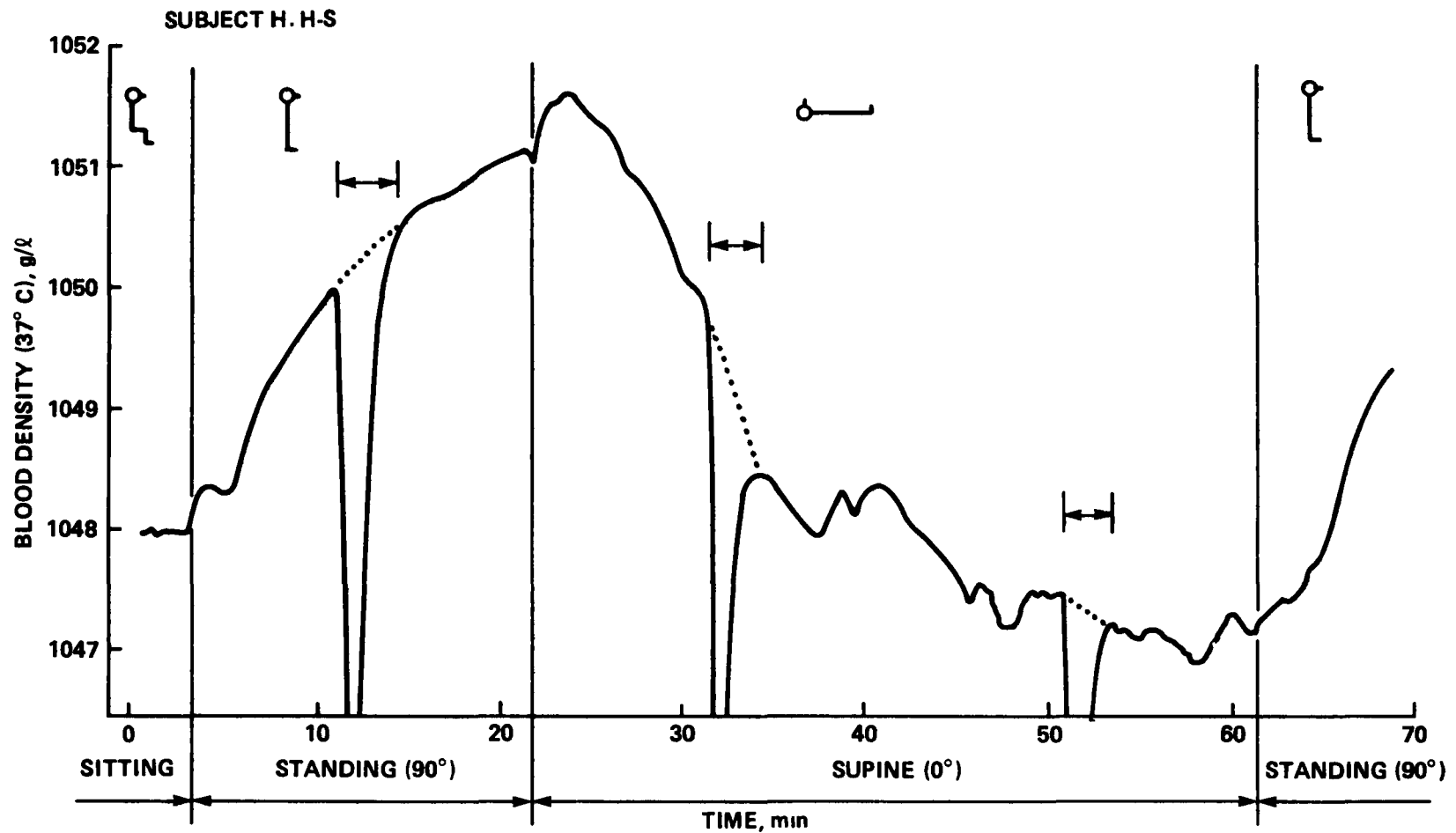


Figure 1.- Continuous blood density recording in a man sitting, standing, and lying supine. The discontinuity (arrows) occurred when the line was flushed with heparinized isotonic sodium chloride solution (density: 999.5 g/l at 37°C). The flow rate was 1.5 ml/min, and the sample volume in the oscillator was 0.1 ml.



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16 Abstract  Detailed descriptions of methods for measuring absolute levels and changes in plasma volume are presented along with derivations of pertinent equations. Reduction in variability of the Evans blue dye dilution technique using chromatographic column purification suggests that the day-to-day variability in the plasma volume in humans is less than $\pm 20$ ml. Mass density determination using the mechanical-oscillator technique provides a method for measuring vascular fluid shifts continuously for assessing the density of the filtrate, and for quantifying movements of protein across microvascular walls. Equations for the calculation of volume and density of shifted fluid are presented.					
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